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Using DNA barcodes to confirm new records of Amazon longfin herrings *Pellona castelnaeana* Valenciennes, 1847 and *Pellona flavipinnis* (Valenciennes, 1837) (Clupeiformes: Pristigasteridae) in the Branco River sub-basin

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Abstract
This note demonstrates the use of a DNA barcoding methodology in confirming new occurrence records of *Pellona castelnaeana* and *Pellona flavipinnis* in the Branco River sub-basin. The DNA barcode result was verified by identification based on morphological characters of both species. Thus, these records increase the species' ranges by more than 600 km in the Amazon and show evidence of high genetic variability in *P. flavipinnis*.

Key words
Amazon basin distribution; range extension; new records; apapás; barcoding; morphological characters.

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Introduction

Fishes of the genus *Pellona*, commonly known as "apapás" or "sardas" in Brazil, are included in the family Pristigasteridae (longfin herrings). Two species have a wide distribution in the Amazon basin: *Pellona castelnaeana* Valenciennes, 1847 and *Pellona flavipinnis* (Valenciennes, 1837). Their general geographic distribution was presented by Whitehead (1985), while more up-to-date occurrence records can be accessed at the Global Biodiversity Information Facility (http://www.gbif.org/). Nevertheless, these sources do not indicate records of these fishes from some important river systems of the Amazon basin, including the Branco River sub-basin in Roraima state. There are, however, personal observations reported by Ferreira et al. (2007). With this

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Figure 1. Specimen vouchers from Branco River sub basin deposited in the Instituto de Pesquisas da Amazônia (INPA) collection: (**a**) *Pellona castelnaeana*. (**b**) *Pellona flavipinnis*.

note we establish occurrence records of *P. castelnaeana* and *P. flavipinnis* (Fig. 1a and 1b, respectively) in the Branco River.

Methods

Individuals of *Pellona* were collected using gill nets (stretched mesh 30–35 mm) during field expeditions to the following localities: upper Branco River (02°47′57.34″ N, 060°40′00.32″ W); middle Branco River (01°38′55.95"' N, 061°13′13.50″ W); and lower Branco River (00°58′06.51″ S, 061°53′58.69″ W) (Fig. 2a; Table 1). Tissue samples were obtained by removing part of the pectoral fin; these were preserved in 95% ethanol, then deposited in the Coleção de Tecidos de Genética Animal (CTGA) at the Universidade Federal do Amazonas (UFAM). The specimen vouchers were fixed in 10% formalin and preserved in 70% ethanol, and were later deposited in the ichthyological collection at the Instituto Nacional de Pesquisas da Amazônia (INPA) under the accession numbers 46381- 46383 (Fig. 1). All sampling was carried out under the ICMBio/IBAMA permit No.11325-1 to IPF.

To confirm the identification of the specimens, we assessed meristic characters commonly used in the identification of these species: the number of the post-ventral scales and the number of the lower-arch gillrakers (Santos et al. 2007). For molecular identification, we used the cytochrome *c* oxidase subunit I (COI, DNA barcoding sensu Hebert et al. 2003). As reference sequences from Amazon-sourced *Pellona* were not available on GenBank, we used samples of *P. castelnaeana* and *P. flavipinnis* caught from known localities (Coari and Manaus, respectively; Fig. 2a) as reported by Whitehead (1985) (Table 1). The samples of *P. castelnaeana* and *P. flavipinnis* from known localities are from unpublished data from Ximenes (2014) and were used here to compare with specimens from Branco River (Fig. 2a). The sequence data were deposited in GenBank under the following accession numbers: KX462719 to KX462723 for *P. castelnaeana* and KX462724 to KX462728 for *P. flavipinnis*.

DNA was extracted using a standard phenol/chloroform protocol (Sambrook et al. 1989), and its quality was visualized on a 1% agarose gel and quantified using a NanoDrop 2000 UV-Vis spectrophotometer. The primers used and the amplification of COI was conducted as described by Ivanova et al. (2007). The PCR products were purified using EXO-SAP (Exonuclease -Shrimp Alcaline Phosphatase) and subjected to fluorescent dye-terminator (ddNTP) sequencing following the manufacturer's recommended protocol for BigDye sequencing chemistry (Life Technologies). Sequences were aligned using the Clustal W algorithm (Thompson et al. 1994) as implemented in BioEdit (Hall 1999).

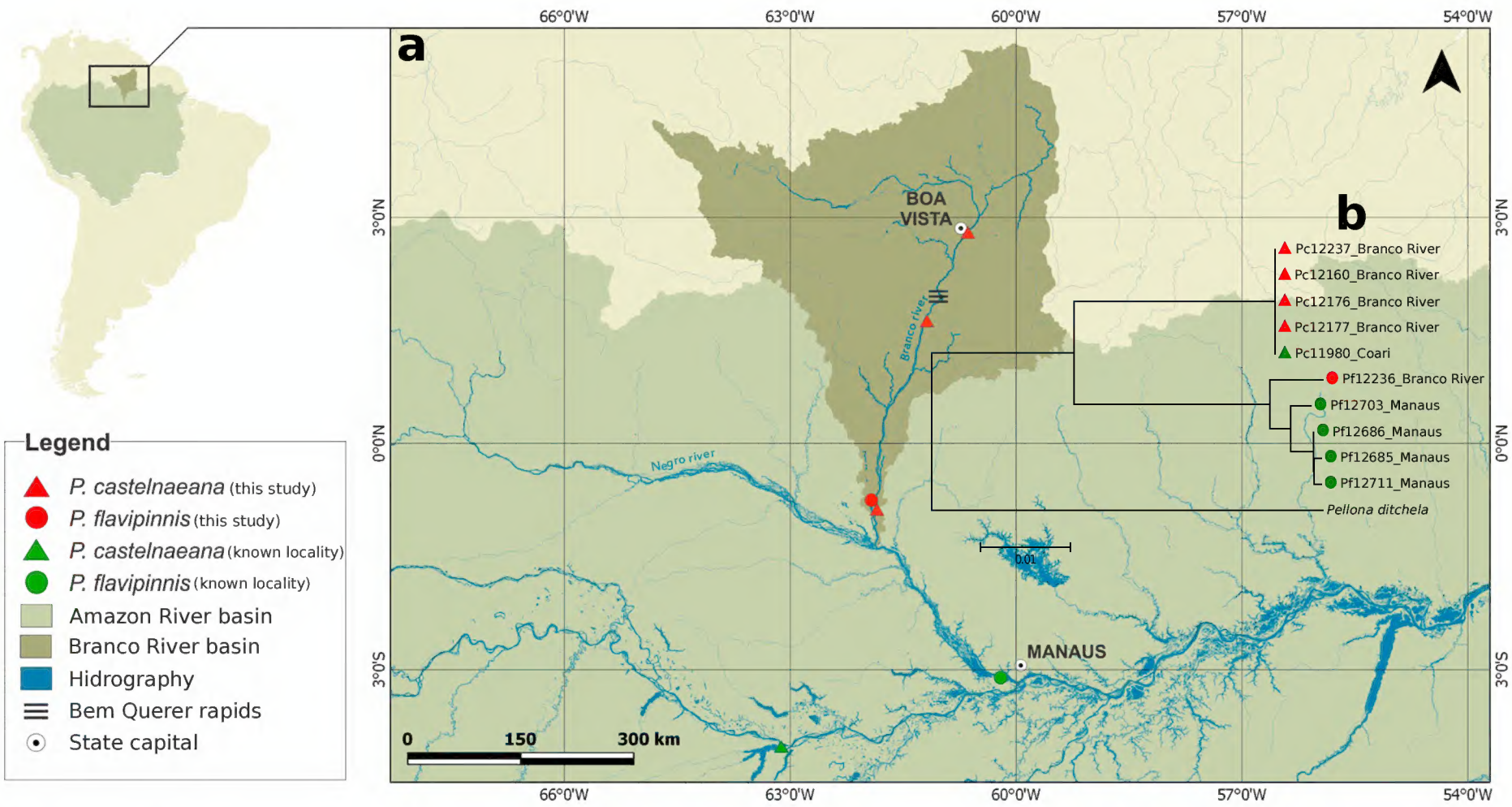

Figure 2. (**a**) Map showing sampling localities for *Pellona flavipinnis* and *Pellona castelnaeana* in the Branco River (red triangle and circle: Boa Vista, Caracaraí and lower Branco River, respectively), as well as individuals that comprise the reference populations for the molecular identifications (green triangle and circle). (**b**) Neighbor-joining tree based on the K2P (substitution model) distances for molecular identification of the *Pellona* species from the Branco River sub-basin. *Pellona ditchela* was used as outgroup.

To visualize the relationships among specimens and clusters, we generated a distance-based tree using the neighbor-joining (NJ) method and the Kimura 2-Parameter (K2P) substitution model, using the software MEGA 6.0 (Tamura et al. 2013). Bootstrap resampling (Felsenstein 1985) was applied to assess the support for individual nodes using 1,000 pseudo-replicates. The intra and interspecific genetic distances were inferred based on the Ward (2009) standard parameters for DNA barcoding of fishes. The marine pellona *Pellona ditchela* (GenBank accession number AP011609) was used as outgroup.

Results

Four specimens of *P. castelnaeana* were collected from the Branco River, along with 1 reference specimen from Coari; one specimen of *P. flavipinnis* was collected from the Branco River, along with 4 reference specimens from Manaus. For both species the NJ tree shows individuals collected from the Branco River grouped with individuals from the reference populations (Fig. 2b). The maximum intraspecific genetic distances obtained were 0% for *P. castelnaeana* (mean 0%) and 1.2% for *P.*

Table 1. Individuals used in this study (ID code used in the map, Figure 2). Voucher and tissue sample codes, sampling localities, site localities, GenBank accession numbers for all the sequences and references. In bold are the new records of this study.

Species (ID)	Voucher	Tissue sample	Sampling localities	Site		GenBank accession	Reference
				Latitude	Longitude		
***Pellona castelnaeana* (Pc12237_Branco River)**	46381	CTGA_12237	Boa Vista city	02°47′57.34″ N	060°40′0.32″ W	KX462719	Present study
***Pellona castelnaeana* (Pc12160_Branco River)**	46381	CTGA_12160	Boa Vista city	02°47′57.34″ N	060°40′0.32″ W	KX462720	Present study
***Pellona castelnaeana* (Pc12176_Branco River)**	—	CTGA_12176	Caracaraí town	01°38′55.95″ N	061°13′13.50″ W	KX462721	Present study
***Pellona castelnaeana* (Pc12177_Branco River)**	46382	CTGA_12177	Lower Branco River	01°03′28.7″ S	061°51′46.1″ W	KX462722	Present study
***Pellona flavipinnis* (Pf12236_Branco River)**	46383	CTGA_12236	Lower Branco River	00°58′06.51″ S	061°53′58.69″ W	KX462724	Present study
Pellona castelnaeana (Pc11980_Coari)		CTGA_11980	Coari town	04°04′37.82″ S	063°8′56.66″ W	KX462723	Present study
Pellona flavipinnis (Pf12703_Manaus)		CTGA_12703	Manaus city	03°04′32.55″ S	060°11′52.99″ W	KX462728	Present study
Pellona flavipinnis (Pf12686_Manaus)		CTGA_12686	Manaus city	03°04′32.55″ S	060°11′52.99″ W	KX462726	Present study
Pellona flavipinnis (Pf12685_Manaus)		CTGA_12685	Manaus city	03°04′32.55″ S	060°11′52.99″ W	KX462725	Present study
Pellona flavipinnis (Pf12711_Manaus)		CTGA_12711	Manaus city	03°04′32.55″ S	060°11′52.99″ W	KX462727	Present study

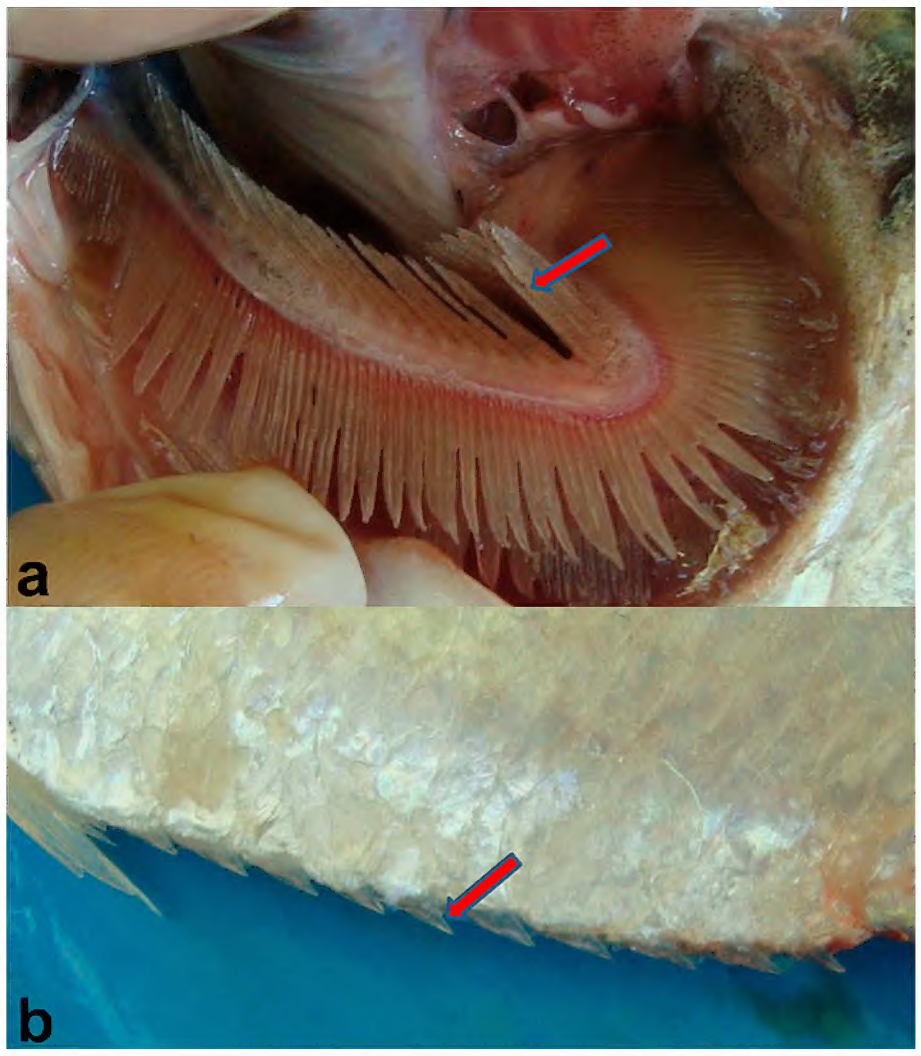

Figure 3. Visual evidence of meristic characters used for *Pellona* species identification: (**a**) lower arch gillrakers, (**b**) post-pelvic scutes.

flavipinnis (mean 0.6%); the minimum interspecific distance between the two was 4.9% (Table 2). These results are in agreement with the barcoding parameter for fishes (Ward, 2009), which suggest a limit up to 2% (maximum intraspecific distance) to consider several individuals belonging to the same species.

Morphological characters, however, can be used to confirm that the specimens collected in the Branco River belong to the species *P. castelnaeana* and *P. flavipinnis*. *Pellona castelnaeana* have 10–11 post-pelvic scutes, 11–13 lower-arch gill-rakers, and yellow coloration, while *P. flavipinnis* have 11–14 post-pelvic scutes, 25–29 lower-arch gill-rakers, and a whitish silver coloration (Fig. 3).

Discussion

One interpretation for the presence of the *P. castelnaeana* and *P. flavipinnis* in the Branco River is based on their migratory life history. The apapás undertake migration for spawning in headwater tributaries (Ikeziri et al. 2008, Le Guennec and Loubens 2004) and mainly use white waters for this purpose because they are rich in nutrients and primary productivity (Barthem and Goulding 2007). The Branco River is considered an important white-water Amazon tributary, and most likely, these fishes are using the river for spawning.

We can conclude that the individuals collected in the Branco River sub-basin correspond to the species *P. castelnaeana* and *P. flavipinnis*, thus expanding the occurrence area for both species by approximately 650 km north of the Amazon basin. Additionally, given the high levels of exploitation of apapás by commercial fisheries (Ikeziri et al. 2008), we consider that ecological and genetic studies on these species are needed to reveal their population dynamics, population structure, and wider taxonomic status. These studies are especially important for *P. flavipinnis*, whose diversity may be underestimated, since our Branco River individual represented a single haplotype with divergence up to 1.2% from the Manaus reference sequences.

The DNA barcoding methodology was shown to be efficient for the identification of *Pellona* species, and can be considered an additional tool for confirmation of new

Table 2. Estimates of pairwise genetic distances between *Pellona* species under the K2P substitution model. Pc: *Pellona castelnaeana*, Pf: *Pellona flavipinnis*. Coari and Manaus represent reference populations (known localities) of the distribution of the *Pellona* species in the Amazon region. *Pellona ditchela* was used as an external reference.

		1	2	3	4	5	6	7	8	9	10	11
1	Pc12237_Branco River (GenBank accession KX462719)											
2	Pc12160_Branco River (GenBank accession KX462720)	0.0000										
3	Pc12176_Branco River (GenBank accession KX462721)	0.0000	0.0000									
4	Pc12177_Branco River (GenBank accession KX462722)	0.0000	0.0000	0.0000								
5	Pc11980_Coari (GenBank accession KX462723)	0.0000	0.0000	0.0000	0.0000							
6	Pf12236_Branco River (GenBank accession KX462724)	0.0498	0.0498	0.0498	0.0498	0.0498						
7	Pf12686_Manaus (GenBank accession KX462726)	0.0489	0.0489	0.0489	0.0489	0.0489	0.0114					
8	Pf12711_Manaus (GenBank accession KX462727)	0.0498	0.0498	0.0498	0.0498	0.0498	0.0123	0.0008				
9	Pf12703_Manaus (GenBank accession KX462728)	0.0489	0.0489	0.0489	0.0489	0.0489	0.0098	0.0048	0.0056			
10	Pf12685_Manaus (GenBank accession KX462725)	0.0498	0.0498	0.0498	0.0498	0.0498	0.0123	0.0008	0.0016	0.0056		
11	*Pellona_ditchela* (GenBank accession AP011609)	0.0817	0.0817	0.0817	0.0817	0.0817	0.0875	0.0850	0.0862	0.0853	0.0862	

records for other Amazonian taxa, but should be verified against morphological data.

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Authors' Contributions

AMX, VNM and JGF collected samples in the field; AMX and VNM identified the specimens and collected data in the lab; AMX and JGM performed data analyses; AMX, VNM, JGM and JGF wrote the NGD; IPF contributed materials and reagents; all authors read, revised and approved the manuscript.

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